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TITLE: Developing Treatment, Treatment Validation, and Treatment Scope in the Setting of an Autism Clinical Trial

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14. ABSTRACT

We obtained IRB approval on December 7th, 2009. We applied for and received a COC from the NIH as required and received final approval from our IRB on March 25th 2010. The test material DHA and the placebo were acquired from Martek with a significant and unforeseen delay August 20th 2010 (please see Partnering project W81XWH-08-1-0730, Task #2). The Data Safety Monitoring Board and all other requirements for subject recruitment have been completed. Additional sources of subjects are being arranged to make up for delays occurring in obtaining IRB approval and issues obtaining the material from Martek (please see Partnering project W81XWH-08-1-0730, Tasks #1 and #2). We have established a set of LC-MSMS conditions appropriate for looking the presence of LXA4, RvD1, RvE1 and Maresin in urine. The necessary standards in natural and deuterated form have been prepared by total organic synthesis. We have established a set of LC-MSMS conditions appropriate for looking the presence of LXA4, RvD1, RvE1 and Maresin in urine.
The SOW was updated on March 22nd 2010.
Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730

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Introduction:

This project is to test to see if DHA treatment can beneficially affect excretion of urinary biomarkers of oxidative stress and the autism clinical phenotype. In addition polymorphic variants of genes of certain enzymes that synthesize and metabolize docosahexaenoic acid (DHA) may contribute to the phenotype of some autism cases. We will test to see if any of these genes are risk factors for autism. We will also measure changes in excretion of the polyunsaturated fatty acid (PUFA) derived biomarkers of oxidative stress (isoprostanes and neuroprostanes) together with the changes in production of anti-inflammatory lipid mediators. We will test these biomarkers to see if we can monitor and validate effectiveness of DHA therapy. We will also test the genotypes of key DHA-metabolizing enzymes can predict which patients will respond to therapy Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730.

Body:

PROJECT #2: PI T.P. STEIN, PhD, PARTNERING PI, W81XWH-08-1-0729

Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730.

Unless otherwise stated, tasks are divided between the synthetic core (directed by Dr. B.W. Spur) and the Analytical core (directed by Dr. T.P. Stein).

Task #1 Obtain IRB approval (Drs. Stein and Spur).

IRB Approval has been obtained.

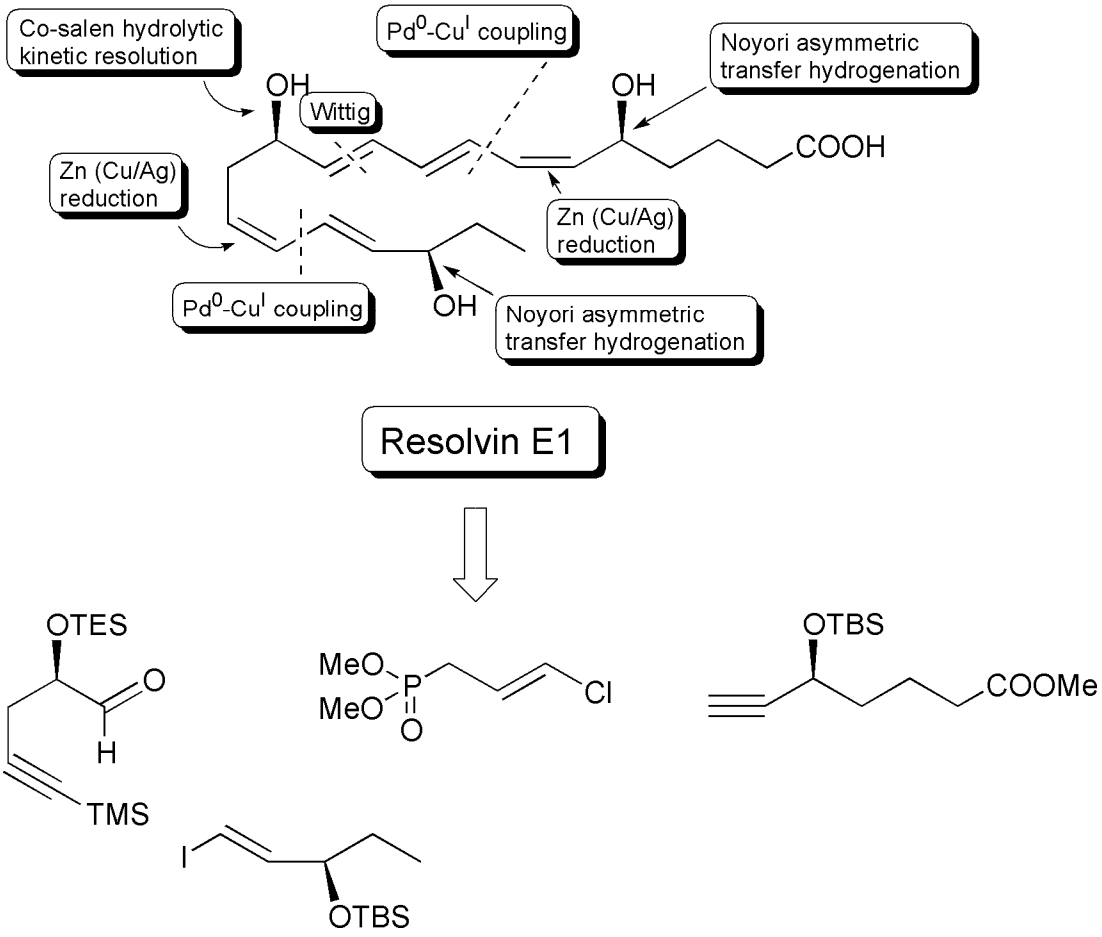
Please see partnering project W81XWH-08-1-0730 Tasks #1 and 2 for detailed description. In brief, the second year of the project has been used to obtain IRB approval from the UMDNJ-RWJMS IRB office. We obtained IRB approval on December 7th, 2009. Our approved protocol and supporting documents were submitted to the Human Research Protection Office (HRPO) Office of Research Protections (ORP) of the DOD for review. We applied for and received a Certificate of Confidentiality from the NIH as per our IRB requirements. The test material DHA and the placebo were acquired from Martek with a significant and unforeseen delay. (Please see Task #2 in partnering project W81XWH-08-1-0730). Subject recruitment has been organized. A Continuing Review has been applied for. The Data Safety Monitoring Board (DSMB) has been set up and provided with necessary documents to hold their initial meeting with the exception of the CR which will be sent as soon as we receive. Human Subjects recruitment will begin once the DSMB have given approval.

Task #2 **After IRB approval has been obtained and permission given to proceed we (B.W. Spur and A. Rodriguez) will chemically synthesize the protective lipid metabolites: (i) Lipoxin A4 (LXA4, 5S, 6R,15S-trihydroxyl-7, 9,13-trans-11-cis-**

eicosatetraenoic acid), (ii) lipoxin A4 precursor, 15-hydroxy-eicosatetraenoic acid, (iii) Resolvin D1 (RvD1, 7S,8R,17S trihydroxy-4Z,9E,11E,13Z,15E, 19Z-docosahexaenoic acid) (iv) Resolvin D precursor (17-hydroxy-docosahexaenoic acid), (v) Resolvin E1 (RvE1, 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16 Eicosapentaenoic acid and (vi) Protectin D1 (10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E, 15Z,19Z-hexaenoic acid) all with and without a deuterium label. It is expected that task #2 will take two years.

1. SYNTHESIS OF Resolvin E1

Dr. Spur is responsible for step (1), synthesis of Resolvins with and without deuterium labels to be used for methods development. Since the last progress report, Dr. Spur has completed the synthesis of Resolvin E1. An outline of the synthesis is described below.

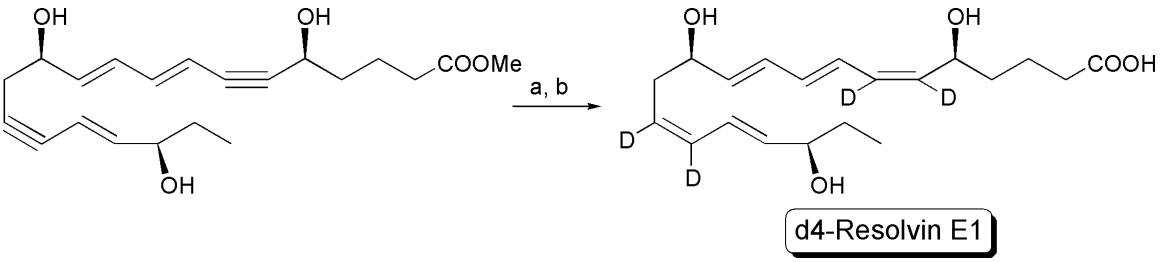


The synthesis of Resolvin E1 was achieved outlined above. The chiral center in the 5- and 18-position were obtained via Noyori asymmetric transfer hydrogenation and the chiral hydroxy group in 12 position resulted from a Jacobsen asymmetric hydrolytic

kinetic resolution. The carbon skeleton was constructed via Sonogashira coupling followed by an Wittig-Horner and finished by a second Sonogashira coupling to produce the di-acetylenic Resolvin E1 precursor. Final freshly prepared Zn/Cu/Ag alloy reduction of the triple bond precursor with H_2O in methanol gave Resolvin E1 methyl ester. Mild ester cleavage with 1N Lithium hydroxide in Water/ Methanol at 0 °C followed by acidification with a saturated solution of NaH_2PO_4 in the presence of Ethyl acetate gave the required Resolvin E1. (Characterized by ^{1}H-NMR, ^{13}C-NMR, UV, MS)

2. SYNTHESIS OF tetra-deutero-Resolvin E1

Since the last progress report, Dr. Spur has completed the synthesis of tetra-deutero-Resolvin E1. An outline of the synthesis is described below.



Reagents and conditions: (a) Zn(Cu/Ag), d4-MeOH, D_2O, 40 °C; (b) 1N LiOH, H_2O, MeOH, 0 °C, then H^+ (NaH_2PO_4 saturated), 0 °C.

The synthesis of the Tetra-deuterated Resolvin E1 was achieved starting from the triple bound precursor as outlined above. Reduction of the triple bond precursor with D_2O in d4 methanol with the freshly prepared Zn/Cu/Ag alloy gave Tetra-deutero Resolvin E1 methyl ester. Mild ester cleavage with 1N Lithium hydroxide in Water/ Methanol at 0 °C followed by acidification with a saturated solution of NaH_2PO_4 in the presence of Ethyl acetate gave the required d4-Resolvin E1. (Characterized by by ^{1}H-NMR, ^{13}C-NMR, UV, MS)

Task #3 **(i) Year 1 and the first half of year two will be spent in developing isotope dilution LC-MSMS assays for the compounds referenced in task #2 (T.P. Stein and technician). Also to be done during that year are to replicate in our laboratory published LC-MSMS assays for isoprostane and isoprostane metabolites in urine, 2,3 dinor-5,6 dihydro-PGF2t and iPF4α-VI (T.P. Stein and technician).**

Dr. Stein is responsible for the analytical aspects. Please note that actual work on this project was precluded by the lack of IRB approval. Any work done was done as part of the new equipment installation program using funds provided by the university and other grants.

The objective of this years research was to determine the baseline analytical conditions for detecting the mediators of interest in the urine. They are Resolvin D1, Resolvin E1, Lipoxin A4, Maresin and Neuroprotectin/Protectin. Dr. Spur has now provided us with labeled and unlabeled Resolvin D1 (RvD1, $^{2}H_2$RvD1), Resolvin E1 (RvE1, $^{2}H_4$RvE1), Lipoxin A4, (LXA4, $^{2}H_2$ LXA4).

During the past year, we have accomplished three goals.

1. To determine optimal LC conditions for separating RvD1, RvE1. LXA4 and 12-HETE.

2. Identify suitable MRM (molecular reaction mechanism) transitions for the quantification of RvD1, RvE1. LXA4 and 12-HETE

The results are shown in the table below.

COMPOUND	**RUN TIME**	**MRM 1**	**MRM 2**	**MRM 3**	**LABELED STD MRM**
RvE1	14.83	349.3>195.2	349.3>129.2	349.3>161.2	
$^{2}H_2$RvE1					353.3>197.2
LXA4	16.32	351.3>217.3	351.3>235.3	351.3>161.2	
$^{2}H_2$LXA					356.3>222.3
RvD1	16.80	375.2>233.1	375.2>141.1	375.2>215.2	
$^{2}H_2$RvD1					377.3>235.1
12-HETE	20.95	319.3>179.2	none	None	
d8-12 HETE					327.3>184.0
MEHP	19.14	277.2>134.1	None	None	
13C-MEHP					281.2>137.1

As can be seen form the table, no problems were encountered in determining suitable LC conditions, fragmentation voltages, collision frequencies of LC conditions.

Note that the table includes two other compounds 12-HETE and MEHP. As part of our analytical methodology we intend to use two internal standards. The first one is 12-Hydroxyeicosatetraenoic acid (12-HETE, $^{2}H_8$ 12-HETE) is used by others as internal standard for LC-MSMS analyses of polyhydroxylated polyunsaturated fatty acids. It was purchased from Cayman Chemicals, (Midland, MI). The second is methylethylhexyl phthalate (MEHP). Many metabolites are excreted in the urine in the free and conjugated to glucose as the glucuronide. It may well be that all of the metabolite of interest is conjugated, some is, or none is. There is no published information as to whether any or some of the compounds of interest are excreted in the free, conjugated or a mixture of both forms. Therefore it is necessary to measure both free and

conjugated (glucuronidated) metabolites. Any glucuronidated compounds in the urine are converted to the free compound by treatment with glucuronidase. To make sure this reaction worked, we will also assay methylethylhexyl phthalate (MEHP) in the free and conjugated (glucuronidated).

We selected MEHP as the internal standard for assessing the de-glucuronidation reaction for three reasons. (i) Conjugated and unconjugated MEHP are always found in human urine (1). They are derived from the ubiquitous phthalate plasticizers. (ii), we have an NIH grant to study phthalate excretion and so are very familiar with the methodology and the behavior of MEHP and (iii) MEHP elutes as a very clean peak at a convenient time under the conditions we have found to give the best resolution of the bioactive lipid mediators.

Task #4 **Use the newly developed assays from task #3 to measuring the markers (isoprostane and it's metabolite, LXA4, RvD1, RvE1 and Protectin), in the urines collected as part of the clinical trial. We anticipate 66 (placebo-treated) and 66 (DHA-treated) subjects; initially two urines pre-treatment and two at the end of the treatment phase will be analyzed for each metabolite. This task will be started one year after the start of patient sample collection. We anticipate having to do multiple LC-MSMS injections because while sample preparation will be common, LC conditions for resolution may well be different. We aim to do the analyses in not more than three batches, resolving several but not all of the compounds in each run (T.P. Stein and technician).**

This task will not begin until subject recruitment, enrollment and treatment have been completed.

Task 5
Data will be collected and analyzed (6-36 months, S Buyske).

This task will not begin until subject recruitment, enrollment, treatment and analysis have been completed.

<u>Task 6</u>
Manuscripts prepared and submitted for publication (03 year, all investigators)

This task will not begin until subject data analysis has been completed.

Key Research Accomplishments

We have established a set of LC-MSMS conditions appropriate for looking the presence of LXA4, RvD1, RvE1 and Maresin in urine,

Reportable Outcomes:

There are no reportable outcomes at this time.

Conclusion:

We are making good progress with the necessary organic syntheses (task #2). As stated in the prior progress report we expect these tasks to be be completed within 15 months. This includes making the corresponding deuterated analogs. Most of these are new and original syntheses, but we believe our timeline for the syntheses to be realistic. Secondly, we now have a basic LC-MSMS analytical program to begin to see which of the bioactive mediators can be detected in urine. As before we aim to complete methods development between months 12 to 18 of this project. Once the methods are in place it should be a relatively simple project to run the patient samples. Our intent, like those of the LC-MSMS papers we are following is to do all of the required analyses in a single run.

In summary, paradoxically the delay in obtaining IRB approval has served us very well. Had we started on time last year we would have been locked into to trying to modify state of the art analytical methodology (LC-MSMS based) to our older GC-MS methodology with no guarantee of success. Thanks to the generosity of the Dean of UMDNJ-SOM we now have state of the art instrumentation and this greatly increases the probability of success for this project.

Appendices:

There are no appendices.